Tuning Differentiation Signals for Efficient Propagation and In Vitro Validation of Rat Embryonic Stem Cell Cultures

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Abstract:	The rat is one of the most commonly used laboratory animals in biomedical research and the recent isolation of genuine pluripotent rat embryonic stem (ES) cell lines has provided new opportunities for applying contemporary genetic engineering techniques to the rat and enhancing the use of this rodent in scientific research. Technical refinements that improve the stability of the rat ES cell cultures will undoubtedly further strengthen and broaden the use of these stem cells in biomedical research. Here, we describe a relatively simple and robust protocol that supports the propagation of germ line competent rat ES cells, and outline how tuning stem cell signalling using small molecule inhibitors can be used to both stabilise self-renewal of rat ES cell cultures and aid evaluation of their differentiation potential in vitro.

Tuning differentiation signals for efficient propagation and *in vitro* validation of rat embryonic stem cell cultures.

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Running title: Efficient propagation of rat embryonic stem cells

Summary

The rat is one of the most commonly used laboratory animals in biomedical research and the recent isolation of genuine pluripotent rat embryonic stem (ES) cell lines has provided new opportunities for applying contemporary genetic engineering techniques to the rat and enhancing the use of this rodent in scientific research. Technical refinements that improve the stability of the rat ES cell cultures will undoubtedly further strengthen and broaden the use of these stem cells in biomedical research. Here, we describe a relatively simple and robust protocol that supports the propagation of germ line competent rat ES cells, and outline how tuning stem cell signalling using small molecule inhibitors can be used to both stabilise self-renewal of rat ES cell cultures and aid evaluation of their differentiation potential *in vitro*.

Key words: rat, embryonic stem cell, tissue culture, derivation, propagation, signalling, differentiation

Introduction

The rat has many characteristics that make it a preferred laboratory animal in biomedical research. Since its domestication over 100 years ago, more than 500 strains of rats have been established, representing a rich resource with which to explore genetics underpinning normal physiology and disease processes in mammals (including humans) [1]. Compared with the mouse, the larger size of the rat means it is more amenable to experimentation. Its physiology is also closer to that of humans, and most strikingly its behavioural and cognitive characteristics make this rodent particularly useful in studying neurobiology and behaviour.

Prospects for designing bespoke genetic models for the study of normal physiology and disease in the rat were, until relatively recently, largely restricted to standard *in ovo* microinjection of randomly integrated transgenes [2]. However, the development of a novel medium formulation for culturing undifferentiated mouse ES cells, where two small molecules are deployed to block critical differentiation regulating pathways (2i culture), reinvigorated efforts to isolate ES cells from the rat, and led to two laboratories successfully reporting the derivation of authentic rat ES cells in 2008 [3-5]. The cell lines contribute to all tissues in chimaeric rats, and are readily amenable to genetic manipulation by standard gene targeting/homologous recombination approaches [6-8]. However, compared with mouse cell lines, under the standard 2i conditions, rat ES cells are less stable in culture and contribute less effectively to tissues in chimaeric animals, particularly to the germ cell lineage [6,9]. Further refinement of protocols for propagating rat ES cells will increase the utility of these cell lines in transgenesis and their applications in studying mammalian biology. Correspondingly, the development of straightforward assays for routinely assessing quality of rat ES cells will help to identify cell lines that efficiently contribute to chimaeras and reliably deliver germline transmission.

The key small molecules deployed in the 2i-culture system are inhibitors of signalling pathways that control cell proliferation and differentiation decisions throughout embryonic

development and adult life. PD0325901 inhibits MEK a critical component of the FGF/ERK-MAPK differentiation pathway in ESC, whereas CHIR99021 inhibits glycogen synthetase kinase 3 (GSK3), a repressor of WNT/β–catenin signalling [3,10]. The immediate downstream effects of the inhibitors are therefore quite distinct: PD0325901 blocks ERK signalling whereas CHIR99021 boosts β-catenin activity. Since the effects of WNT/β-catenin on cell differentiation can be dose and context-dependent, we investigated the requirement for CHIR99021 in rat ES cells and found that application of an optimal dose of CHIR99021 was necessary to robustly stabilise rat ES cells in culture: too little or too much CHIR99021-induced β-catenin activity leads to differentiation and degeneration of rat ES cell cultures [11]. In contrast, high levels of PD0325901 reduce ES cell growth but do not appear to interfere with self-renewal. A further complication is that the biological activity and purity of the two inhibitors, particularly CHIR99021, can vary between suppliers and between batches [12]. It is clear therefore that identifying the correct dose of the 2i inhibitors is an important consideration when culturing pluripotent rat ES cells.

Here we outline a protocol for propagating germ line-competent rat ES cells, and a simple assay for establishing appropriate levels of the 2i inhibitors. We also describe how simply tuning the levels of the 2i signalling inhibitors can be used to selectively promote differentiation to rapidly evaluate the developmental potential of targeted rat ES cell clones.

2. Materials

2.1 Rat ES cell culture

1. Rat ES cell growth medium: N2B27 medium, 1μM PD0325901, 3μM CHIR99021, 1000U/ml mouse LIF (50U is defined as the concentration of LIF in 1ml medium that induces the differentiation of 50% of M1 myeloid leukemic cells). Filter sterilise using a 0.22μM filter and store at 4^{o}C for up to two weeks.

2. N2B27 Medium: Neurobasal : DMEM/F12 (1:1), 1% N2 supplement, 2% B27 supplement, 2mM L-glutamine, 0.1nM β-mercaptoethanol. Filter sterilise using a 0.22μM filter and store at 4°C for up to four weeks.

3. Neural differentiation medium: N2B27, 0.25μM PD0325901, 1.5μM CHIR99021. Filter sterilise using a 0.22μM filter and store at 4°C for up to two weeks.

4. Muscle differentiation medium: N2B27, 3μM CHIR99021. Filter sterilise using a 0.22μM filter and store at 4°C for up to two weeks.

5. PD0325901 (MEK inhibitor): resuspend to 10mM in DMSO, aliquot and store at -80°C for up to one year. For a working stock solution dilute 10mM stock to 1mM with N2B27 and store at 4°C for up to two weeks.

6. CHIR99021 (GSK3 inhibitor): resuspend to 10mM in DMSO, aliquot and store at -80°C for up to one year. For a working stock solution dilute 10mM stock to 1mM with N2B27 and store at 4°C for up to two weeks (*see* **Note 1**).

7. TVP: 0.025% trypsin, 1mM EDTA, 1% chicken serum in PBS. Filter sterilise using a 0.22μM filter and store at -20°C for up to one year.

8. Freezing medium (2x stock): 20% FCS and 20% DMSO in N2B27 (for rat ES cells) or feeder medium (for feeders). Filter sterilise using a 0.22μM filter and store at 4°C for up to four weeks.

9. Feeder medium: GMEM, 10% FCS, 1x non-essential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 0.1nM β-mercaptoethanol.

10. Blastocyst medium: M2 medium (commercially available) +10% FCS. Filter sterilise using a 0.22μM filter and store at 4°C for up to one week.

11. PBS: prepared from commercially available phosphate buffered saline tablets dissolved in tissue grade water and autoclave sterilised.

12. Gelatin: add gelatin from porcine skin (0.1% w/v) to tissue grade water and autoclave sterilise. For coating of tissue culture plastic add a minimal volume of 0.1% gelatin, incubate at room temperature for two minutes then aspirate.

13. Laminin: aliquot laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane and store at -20^{o}C. For coating of tissue culture plastic dilute a stock aliquot to 10μg/ml in cold PBS (4^{o}C), add a minimal volume to the well then incubate at 37^{o}C for at least two hours. Aspirate the laminin and wash the well three times with PBS. The final PBS wash is left on the well and removed immediately prior to plating the cells.

2.2 Alkaline Phosphatase staining (Sigma)

1. Fixative: 5ml citrate solution, 13ml acetone, 1.6ml formaldehyde (37%). Prepare fresh.

2. Stain: mix 200μl sodium nitrite solution + 200μl FRV-alkaline solution. Incubate two minutes at room temperature then add 9ml sterile distilled water. Add 200μl naphthol AS-BI alkaline solution, mix and use immediately.

2.3 Immunocytochemistry

1. Fixative: dissolve paraformaldehyde (4% w/v) in PBS at 70^{o}C for two hours. Aliquot and store solution at -20^{o}C for up to four weeks.

2. Blocking solution: dissolve bovine serum albumin (1% w/v) and serum (10% v/v) (from the same species as the secondary Ab was raised in) in PBST.

3. PBST: Triton X100 (0.3% v/v) in PBS.

4. Antibodies: βIII-tubulin (1:500, Covance, MMS-435P), skeletal myosin (1:20 A1025, gift from Professor Michelle Peckham, University of Leeds).

3. Methods

3.1 Rat ES cell culture

Rat ES cells are derived and propagated in 2i+LIF medium on a mitotically-inactivated feeder cell layer of mouse embryonic fibroblasts.

3.1.1 Preparation and plating of feeder cell stock

1. Plate day 13.5 mouse embryonic fibroblasts (MEFs) onto a 150cm^2 gelatin-coated tissue culture flask in feeder medium at 1x10^4/cm^2 and incubate at 37^{o}C, 5%CO_2.

2. Replace approximately two-thirds of the medium when the cells are 80% confluent (typically after 48-72 hours).

3. The following day, aspirate the medium, wash the cells twice with 5ml of PBS then add 3ml of TVP and incubate at 37^{o}C, 5%CO_2 for 3 minutes.

4. Disperse to single cell suspension by trituration using a 5ml pipette and transfer to 20ml of feeder medium.

5. Pellet the cells by centrifugation at 600*g* for 3 minutes.

6. Aspirate the supernatant and resuspend the cells in 5ml of feeder medium and count the cells using a haemocytometer.

7. Plate all the cells into the appropriate number of gelatin-coated 150cm^2 flasks as described above (1).

8. Repeat this expansion a total of three times until there are around 27 flasks of MEFs (*see* **Note 2**). Once the final passage of cells is approximately 90% confluent, prepare for γ-irradiation as detailed below.

9. Process the flasks in manageable batches (typically four at a time). For each flask, aspirate the medium, wash twice with 5ml of PBS then add 3ml TVP and incubate 37^{o}C, 5%

CO_2. Disperse to single cell suspension by trituration using a 5ml pipette and transfer to 7ml of feeder medium.

10. Pool the cells from each flask to give a final volume of 40ml then pellet the cells by centrifugation at 600*g* for 3 minutes.

11. Aspirate the supernatant and resuspend the cells in 20ml of feeder medium.

12. Incubate the cells at 37^{o}C, 5% CO_2 until all the flasks have been processed then pellet by centrifugation at 600*g* for 3 minutes.

13. Resuspend and pool all the cells in a final volume of 20ml feeder medium.

14. Mitotically-inactivate the cells by γ-irradiation exposure to 5Gy of Cs137.

15. Freeze the cells in cryovials at 1x10^{6}cells/vial in a total volume of 1ml (500μl cells + 500μl 2x freezing medium). Store at -80^{o}C for up to one month and -150^{o}C for long-term storage.

16. Plate irradiated feeders at a density of 1.5x10^{4}/cm^{2} on to gelatin-coated tissue culture plastic in feeder medium (*see* **Notes 3--4**).

3.1.2 Derivation

1. Harvest rat blastocysts at 4.5 days post-coitum. Remove the uteri and place in PBS on ice.

2. Blot the uterus on a piece of paper towel then remove the ovaries and trim off excess fat using a fine pair of dissecting scissors.

3. Place the uterus into a 5cm plastic petri dish and flush each uterine horn with 0.5-1ml blastocyst medium using a 1ml syringe, and needle with an outer diameter of 25 gauge (*see* **Note 5**).

4. Observe the blastocysts under a low-power microscope and recover using a finely-drawn glass pasteur pipette by mouth pipetting into a fresh drop of blastocyst medium.

5. Remove the zona pellucida by transferring each blastocyst, one at a time, into a drop of acidic Tyrode's solution. Observe the process under a low-power microscope. After several seconds the zona pellucida dissolves. Immediately transfer the de-zonaed blastocyst into a fresh drop of blastocyst medium.

6. Transfer one de-zonaed blastocyst to one well of a feeder-coated 96-well plate containing 200μl 2i+LIF medium.

7. Replace half the volume of medium every second day. After five to six days aspirate the medium carefully from the well and add 50μl TVP. Incubate for two minutes at room temperature then pipette disperse to single cell and transfer to 1ml of 2i+LIF in a 1.5ml tube. Pellet the cells by centrifugation at 600*g* for 3 minutes. Carefully aspirate the supernatant and resuspend the cell pellet in 2i+LIF. Plate all the cells into one well of a feeder-coated 96-well plate in a final volume of 250-300μl 2i+LIF.

8. Expand the established cell line as described in section 3.1.3.

3.1.3 Rat ES cell propagation

1. Aspirate medium and add a minimal volume of TVP to cover the cells (typically 200μl for a 2cm^2 well).

2. Incubate at room temperature for two minutes then disperse to a single cell suspension using a pipette and confirm by observing under a light microscope.

3. Transfer cell suspension to a sterile tube containing 10-20x volume of N2B27 and pellet the cells by centrifugation at 600*g* for 3minutes.

4. Carefully aspirate most of the supernatant and resuspend the cells in 2i+LIF medium to an appropriate density, as recommended by the chosen cytometric method (typically 0.5-1x10^6/ml using a haemocytometer).

5. Plate the cells at a density of 0.5-1x10^5/cm^2 onto a monolayer of γ-irradiated MEFs in 2i+LIF medium (*see* **Notes 6--8**).

6. Feed the next day and repeat passaging every two days. Photographs of typical rat ES cell cultures one day after plating and a day later, when the cells are ready for passaging, are shown in Figure 1.

3.1.4 Freezing rat ES cells

1. Prepare a single cell suspension of cells and pellet as in section 3.1.3

2. Resuspend the cells in 2i+LIF medium and transfer at least 5x10^5 cells to a cryovial in a volume of 500μl.

3. Add an equal volume of 2x Freezing medium, gently pipette to ensure uniform distribution of cells in 1x freezing mix then transfer the vial to a polystyrene storage box and store at -80^{o}C overnight.

4. Store in -80^{o}C for up to one month. If longer-term storage is required, transfer the vial to -150^{o}C within one week.

3.1.5 Thawing rat ES cells

1. Prepare 10ml of warmed (37^{o}C) N2B27 in a 15ml sterile tube.

2. Collect the frozen vial of cells on dry-ice. Place the vial within the culture hood and thaw the cells by transferring warm N2B27 back and forth between the 15ml tube and cryovial until all the cell stock is thawed and transferred into the 15ml tube.

3. Pellet the cells by centrifugation at 600*g* for 3 minutes.

4. Carefully aspirate most of the supernatant and gently resuspend the pellet in 2i+LIF medium.

5. Transfer the cell suspension into a feeder-coated well containing 2i+LIF medium (typically 2-4x10^5 frozen cell stock/cm^2).

6. Recover any left-over cells by rinsing the tube with 200µl 2i+LIF medium and transfer to the same well.

7. Incubate at 37°C, 5%CO_2 overnight and replace with fresh medium the next morning.

3.2.1 Determination of working concentrations of inhibitors for stable rat ES cell growth.

To determine the dilution of the two inhibitors (PD0325901 and CHIR99021) that best maintain rat ES cell growth, serial dilutions of both current and new batches of inhibitors are compared based on their ability to maintain rat ES cell growth in feeder-free conditions on a laminin substrate. The elimination of feeder-support in these assays reliably exposes the acute requirement of rat ES cells for the inhibitors and allows differentiation to be reliably assessed after 5 days of culture.

1. Prepare a serial dilution of the inhibitors (see below).

CHIR99021 concentrations: 6µM, 4µM, 3µM, 2µM, 1µM, 0µM (prepared in N2B27 + 1µM PD0325901 + 1000U/ml LIF).

PD0325901 concentrations: 4µM, 3µM, 2µM, 1µM, 0.5µM, 0µM (prepared in N2B27 + 3µM CHIR99021 + 1000U/ml LIF).

2. Prepare a single cell suspension of rat ES cells as described in section 3.1.3.

3. In order to remove feeder cells plate the suspension onto gelatin-coated tissue culture plastic in 2i+LIF medium and incubate at 37°C, 5%CO_2 for 20 minutes. Within this time the

feeder cells attach to the gelatin-coated tissue culture plastic leaving the medium enriched with floating and loosely attached rat ES cells.

4. Carefully transfer the ES cell-enriched medium to a 15ml tube, leaving a little medium behind to ensure as little feeder cell carry-over as possible, and pellet the cells by centrifugation at 600*g* for 3 minutes.

5. Aspirate the supernatant then resuspend the cell pellet in N2B27 and count using a haemocytometer.

6. Plate the cells at a density of $5x10^3/cm^2$ onto laminin-coated wells into each of the dilutions.

7. Incubate at 37^{o}C, 5% CO_2 for five days then assay cell growth by alkaline phosphatase staining of the cultures. A typical result of an assay where new and current batches of inhibitors have similar biological activities is shown in Figure 2. Note that whereas ES cell colony formation becomes obvious at all PD concentrations above 1μM, maximal self-renewal activity of CHIR lies between 2-3μM, and therefore requires careful titration for optimal results.

3.2.2 Alkaline Phosphatase staining

1. Aspirate the medium from the wells and add a minimal volume of fixative to cover the cells (250μl/cm^2).

2. Incubate at room temperature for 30-60s then aspirate fixative.

3. Wash the cells with sterile distilled water for 30-60s then aspirate.

4. Add a minimal volume of stain to cover the cells (250μl/cm^2).

5. Incubate in the dark at room temperature for 10-20 minutes until colour development is sufficient.

6. Aspirate stain and wash three times briefly with sterile distilled water.

7. Store in last water wash at 4^{o}C. The plate can be scanned or cells photographed using a light microscope.

3.3 Assessment of differentiation potential of rat ES cells

To rapidly evaluate the biological potency of newly derived rat ES cell lines or clones, we routinely use simplified monolayer and embryoid body differentiation protocols.

3.3.1 Tuning signalling to evaluate differentiation potential of rat ES cells

We have found that modulating the ratio of CHI99021 and PD0325901 in the medium on feeder-free cultures can be used to bias differentiation in favour of particular lineages and thereby provide a simple qualitative assessment of the differentiation potential of rat ES cells.

1. Prepare a single cell suspension of feeder-free rat ES cells as described in section 3.2.1.

2. Plate the cells on to laminin-coated tissue culture plastic at a density of 0.5-1x10^{4}/cm^{2} in either neural or muscle differentiation medium.

3. Feed the cells every second day. After five days passage the cells as described in section 3.1.3 and plate on to laminin-coated tissue culture plastic at a density of 0.5-1x10^{4}/cm^{2} in either neural or muscle differentiation medium. Several wells can be plated to accommodate multiple downstream assays.

4. Feed the cells every second day. By day four to six (total of nine to eleven days) overt differentiation can be observed in the culture. Fix the cells for immunocytochemistry or harvest for RT-PCR analysis (Figure 3).

3.3.2 Immunocytochemistry determination of lineage-specific differentiation

1. Aspirate medium from the wells and wash briefly with PBS.

2. Add a minimal volume of fixative and incubate at room temperature for 10 minutes.

3. Aspirate fixative and wash twice, briefly with PBS.

4. Aspirate PBS and wash at room temperature with PBST four times for 5 minutes each with gentle rocking.

5. Aspirate PBST, add blocking solution and incubate at room temperature for two hours.

6. Dilute primary antibody in blocking solution.

7. Aspirate block, add diluted primary antibody and incubate overnight at 4^{o}C.

8. Aspirate primary antibody and wash at room temperature with PBST four times for 5 minutes each with gentle rocking in the dark.

9. Dilute secondary antibody in blocking solution.

10. Aspirate last PBST wash, add diluted secondary antibody then incubate in the dark (fluorescent secondary Ab is sensitive to light) at room temperature for two hours.

11. Aspirate secondary antibody and wash at room temperature with PBST four times for 5 minutes each with gentle rocking.

12. Aspirate last PBST wash and wash briefly with PBS.

13. Aspirate PBS, add DAPI (1:10,000 dilution in PBS) and incubate in dark at room temperature for 5 minutes.

14. Aspirate DAPI and wash three times briefly with PBS. The final PBS wash is left on the cells.

15. The cells are ready to photograph using fluorescent light microscopy or can be stored in the dark at 4^{o}C for up to two weeks.

3.3.3 Embryoid body differentiation

1. Prepare a single cell suspension of rat ES cells in 2i+LIF as described in section 3.1.3.

2. Plate 2x10^{5}/cm^{2} cells onto non-coated, low-adherence plastic wells in 2i+LIF and culture overnight at 37^{o}C, 5%CO_2 (typically in 5ml of medium per well of a 6-well plate)

3. Add 50% more medium to the wells the next day.

4. After a further 24 hours the cells will have aggregated to form early stage embryoid bodies. Transfer all of the medium and embryoid bodies to a sterile 50ml tube and incubate at room temperature for 10 minutes to allow the embryoid bodies to settle to the bottom.

5. Carefully aspirate most of the supernatant then resuspend the embryoid bodies gently in 5ml of medium containing 10% fetal calf serum (feeder medium) using a 5ml pipette (the wide diameter bore prevents disruption of the embryoid bodies) and return to a fresh10cm^{2} non-coated, low-adherence plastic well.

6. After 48 hours in feeder medium the embryiod bodies will have continued to grow, but now appear more irregular. Plate approximately twenty embryoid bodies per well onto a 10cm^{2} gelatin-coated tissue culture well in feeder medium using a 1ml pipette. Plate multiple wells depending on the number of different analyses required.

7. 48 hours post-plating onto gelatin the embryoid bodies should have attached and cells should have started to migrate out from the base of the bodies across the surface of the well.

8. Replace two-thirds of the medium every two days. Monitor the differentiation process daily. Good quality differentiated embryoid bodies should flatten and spread onto the plate but retain a multi-layered core, whilst at their periphery migrating morphologically diverse cell types should be readily visible. Four to nine days after attachment patches of rhythmically beating cells should have formed in many embryoid bodies, signifying the presence of functional, electrically-coupled cardiomyocytes. The embryoid bodies can be photographed, immunostained or harvested for RNA.

4. Notes

1. CHIR99021 forms a white precipitate, giving the solution a turbid appearance. This precipitate may settle during storage to form a white pellet. Resuspend the pellet thoroughly by pipetting prior to use.

2. MEFs will undergo only a limited number of cell divisions before senescing. Therefore, MEFs should only be cultured for 15-20 days from P0 stocks (equivalent of four to five passages) prior to γ-irradiation.

3. The MEFs will take a few hours to attach and spread across the surface of the plate. Therefore, MEFs should be plated the day before or at least four hours prior to use.

4. The maximum shelf life of plated γ-irradiated MEFs is 10-12 days.

5. Take care, when inserting the syringe needle within the uterine lumen that the uterine wall does not tear. If it does ensure that the tip of the needle is inserted beyond the tear. A pair of forceps can be used to grip the uterus around the needle to keep it steady whilst flushing.

This will ensure the medium passes through the entire length of the uterine horn and does not escape through the tear.

6. The optimum density of rat ES cells is 0.5-1x10^5/cm^2. Less than this will encourage differentiation. More than this and the medium will become exhausted.

7. The optimum volume of rat ES cell growth medium and feeding routine should be such that the medium will not become exhausted whilst, at the same time, limits dilution of any autocrine or paracrine self-renewal factors. The cells are fed daily and two hours before passaging. Typically for the optimum plating density (see 4.2 above) 1ml of medium/cm^2 is used and two-thirds of the medium is changed daily. Affirmative

8. Even plating of the cells can be difficult to achieve, particularly in wells smaller than a 6-well. For smaller wells, a 1ml pipette is used to gently disperse the cells. For larger wells the plate can be gently rocked back-and-forth and side-to-side twice.

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Figure legends

Fig. 1.

Optimal and sub-optimal culture of rat ES cells.

(A) Brightfield images of rat ES cells cultured in optimal conditions at day 1 and day 2 post-plating. Cells form compact, rounded colonies. (B) Brightfield image of rat ES cells cultured in sub-optimal plating conditions. Colonies appear irregular or flattened, with overt differentiation. Arrows highlight areas of differentiation. Magnification x100.

Fig. 2.

Assessing the optimal concentration of inhibitors for rat ES cell maintenance.

Alkaline phosphatase stained rat ES cells grown on laminin in different concentrations of PD0325901 (upper panel) and CHIR99021 (lower panel) for five days. The staining compares variation between two inhibitor stocks.

Fig. 3.

Tuning inhibitor concentration for lineage-specific differentiation.

(A) RT-PCR analysis of rat ES cells grown in 3μM CHIR99021 with different concentrations of PD0325901 for 11 days establishes that tuning of MEK inhibition directly influences lineage-specific differentiation. Cells were analysed for ES cell (Oct4 and Nanog), Neural (Pax6) and myogenic (myf5) markers. (B) Immunostaining for βIII-tubulin (left panel) and skeletal myosin (right panel) following differentiation of rat ES cells for 11 days in 1.5μMCH + 0.25μMPD and 3μMCH + 0μMPD respectively. Cells were co-stained using DAPI to identify nuclear DNA. Magnification X100.

Figure 1

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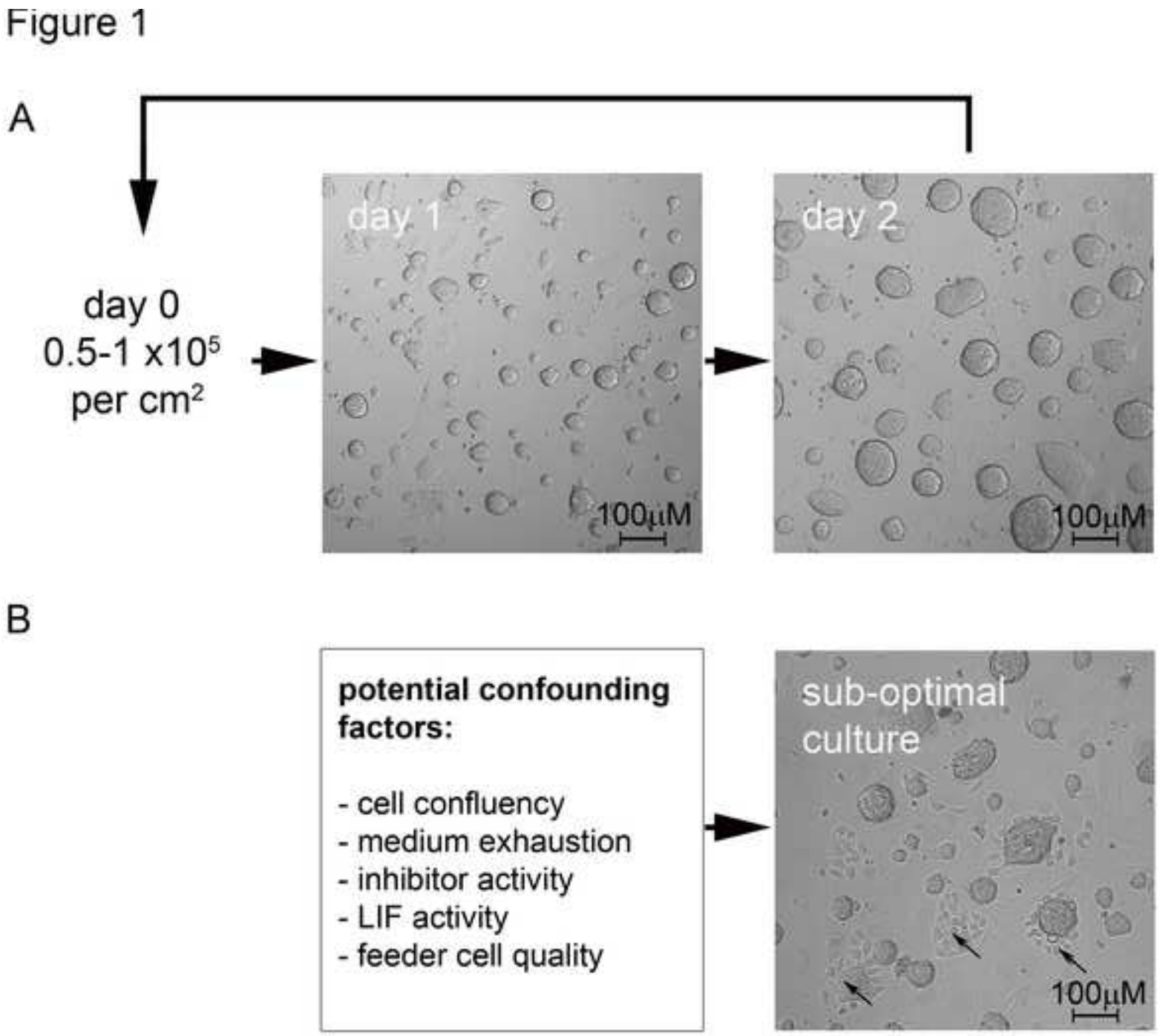

Figure 2
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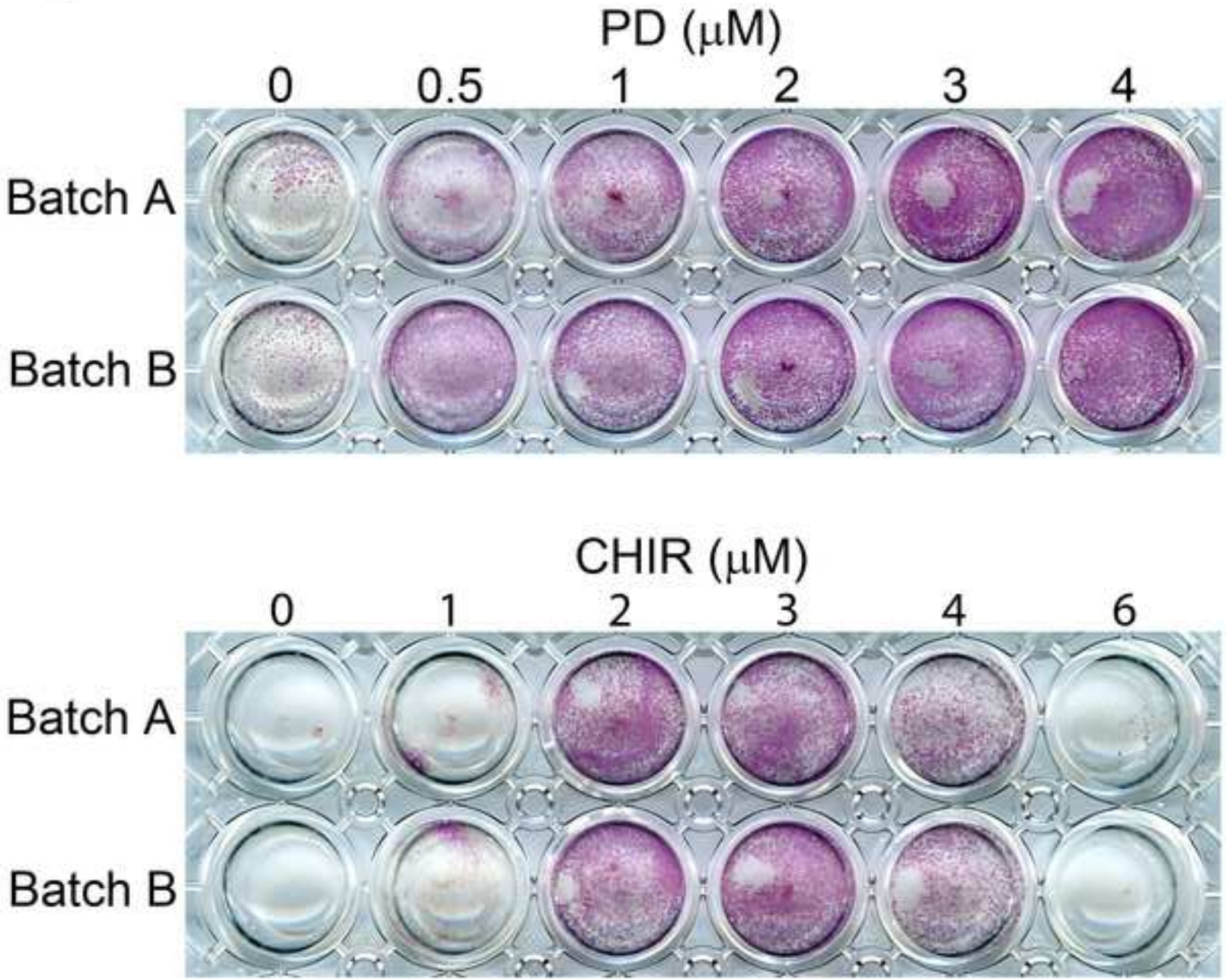

Figure 3
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